NOTE **Clinical Pathology**

Immunohistochemical Localization of Endothelial and Inducible Nitric Oxide Synthase within Neurons of Cattle with Rabies

Taekyun SHIN[1)], Daniel WEINSTOCK[2)], Marlene D. CASTRO[2)], Amir N. HAMIR[3)], Thomas WAMPLER[4)], Mark WALTER[4)], Hyun Young KIM[4)] and Helen ACLAND[4)]

[1)]Department of Veterinary Medicine, Cheju National University, Jeju 690–756, Republic of Korea, [2)]Center for Veterinary Diagnostics and Investigation, Pennsylvania State University, University Park, PA, [3)]NADC, ARS, USDA, Ames, IA, and [4)]Pennsylvania Veterinary Laboratory, Harrisburg, PA, U.S.A.

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ABSTRACT. The expression of constitutive endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) in the brains of cattle with natural rabies was studied. Increased expression of eNOS was detected in neurons of the brain stem and Purkinje cells of cerebellum. By contrast, iNOS was diffusely localized in the cytoplasm of affected neurons, and some inflammatory cells were positive. eNOS and rabies antigen were co-localized in inclusion bodies (Negri bodies) in neurons. The specific localization of eNOS, but not iNOS, in the Negri bodies suggests that eNOS is involved in the formation of rabies virus inclusion bodies.
KEY WORDS: cattle, nitric oxide synthase, rabies.

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In central nervous system (CNS) tissues, all the major nitric oxide synthase (NOS) isoforms are either expressed constitutively or induced by the appropriate stimulation [3]. The constitutive NOSs are most important for the initial generation of NO in the CNS, which is important for neurotransmission. Since iNOS is not expressed in brain cells without immunological stimulation [10, 11], it plays no role in the initial generation of NO. Besides its expected physiological role, NO is likely involved in CNS disorders, including viral encephalitis [1, 6, 8, 9, 14]. Despite certain harmful effects, NO is also a key component in the host defense against a variety of pathogens, including viruses [2, 12]. Inhibitory effects of NOS on the replication of both DNA and RNA viruses have been demonstrated [12].

Rabies virus infections in the brain are characterized histologically by perivascular cuffing with mononuclear cells, the formation of inclusion bodies in neurons [5, 7, 8], neuronal degeneration, and cell death [7, 8]. A significant increase in iNOS expression has been observed in rat brain that was experimentally infected with rabies virus [6, 14]. This suggests that iNOS plays a key role in the neuropathogenesis of rabies via the generation of NO. Conversely, constitutive nNOS expression decreases in the rat brain with experimental rabies infection without a loss of neurons [1]. Although dynamic changes in NOS expression have been identified in experimental rabies infection, little is known about the expression of iNOS and eNOS in natural rabies cases. This study describes the expression of both iNOS and eNOS in the brain of cattle with natural rabies.

Three brains from cows with natural rabies were studied. These cases were selected from the data bank of the Pennsylvania Animal Diagnostic Laboratory System (PADLS) in Harrisburg, Pennsylvania, between 1992 and 1999. Each brain was fixed in 10% neutral buffered formalin and processed using standard histologic techniques. Brains from two cows diagnosed with enteritis were used as neurologically unaffected controls based on the absence of histologic inflammatory lesions.

Immunohistochemical staining was performed using paraffin-embedded tissue sections. The antisera used in this study were anti-rabies monoclonal antibody 802–2[5], rabbit anti-glial fibrillary acidic protein (GFAP) (Sigma, St. Louis, MO), rabbit anti-inducible NOS (Sigma), and rabbit anti-endothelial NOS (Sigma). The immunostaining kit used was the peroxidase-labeled [strept]avidin-biotin (LAB-SA) or alkaline phosphatase-labeled LAB-SA procedure (Zymed Laboratories, Inc., San Francisco, CA). The colorizing substrates were AEC (red) and NBT/BCIP (blue), respectively. To elucidate whether eNOS and rabies virus share common epitopes in inclusion bodies, double staining for both eNOS and rabies virus antigens was performed. Epitope blocking was attempted by pre-incubating the tissue with antiserum to either eNOS or rabies nucleoprotein followed by immunostaining with the other antiserum.

In all three brains, perivascular cuffing was observed in the hippocampus, cerebellum, and pons ventral to the cerebellum. Viral inclusion bodies (Negri bodies) were common in the neurons of the brain stem and in the Purkinje cells of the cerebellum (Fig. 1, A). Viral antigens were distributed in the cytoplasm of neuronal cells and neuronal processes (Fig. 1, B). Inclusion bodies of varying sizes were also positive and usually had a granular staining pattern.

In rabies-infected cows, eNOS was specifically localized in neurons in the brain stem and in Purkinje cells of the cerebellum with viral inclusion bodies (Fig. 1C). The granular staining pattern of eNOS in the neurons was similar to that of rabies antigen (Fig. 1B). This granular distribution of eNOS in the neurons contrasted with the diffuse distribution of iNOS (Fig. 1D). Some endothelial cells and astrocytes reacted weakly to eNOS antibody. In control brains, some vascular endothelial cells and astrocytes were weakly positive for eNOS. There were some round iNOS-positive cells

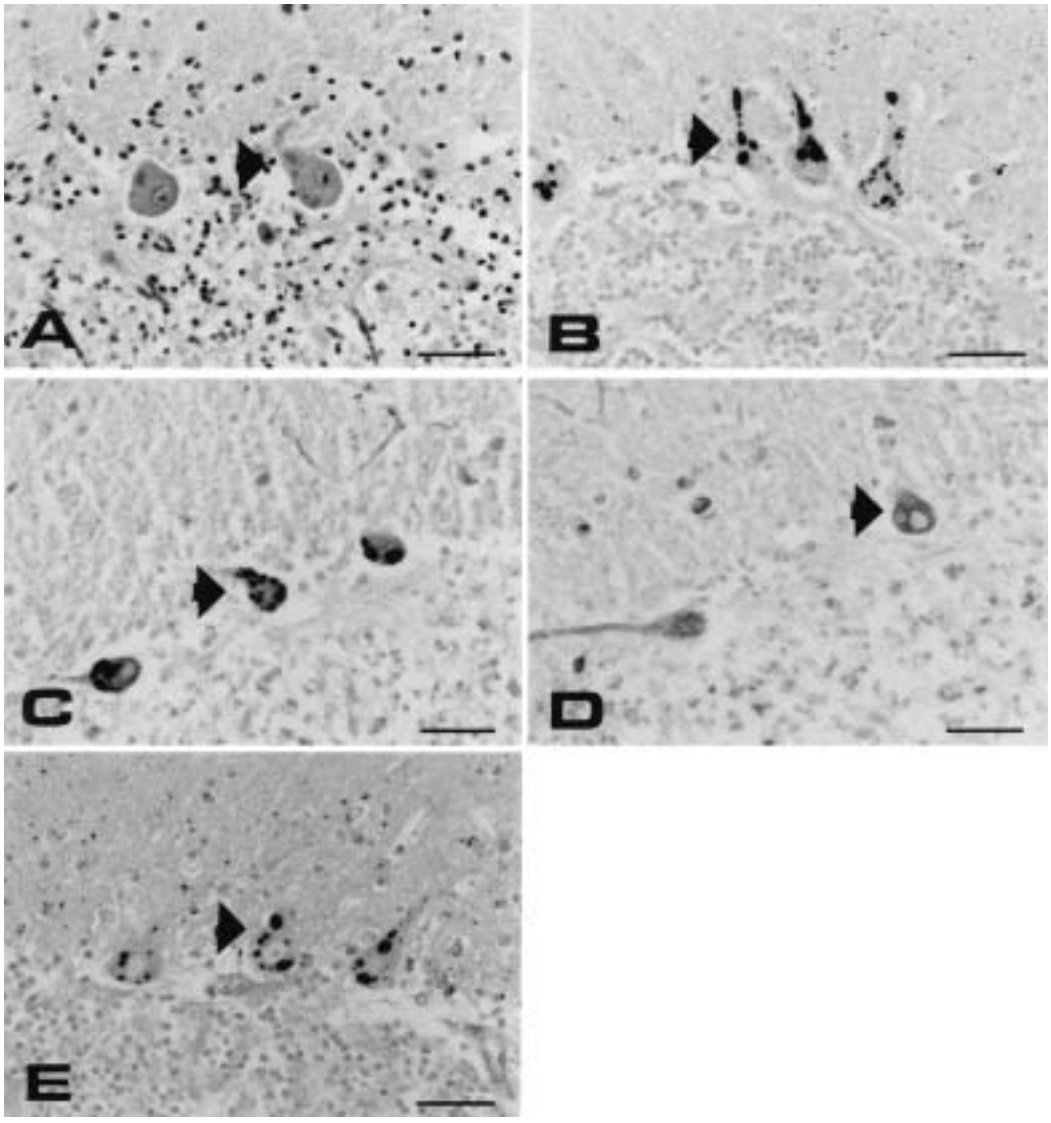

Fig. 1. Histological findings of rabies virus infection in the cerebellum of a cow. A) Typical rabies inclusion bodies (arrowhead) in the Purkinje cells of the cerebellum. B) Granular staining of rabies viral antigen in Purkinje cells (arrowhead). C) The eNOS distribution in the cerebellum was localized in a granular manner that was similar to rabies virus immunoreactivity. D) Diffuse iNOS immunoreactivity detected in the cytoplasm of Purkinje cells in the cerebellum. E) Double labeling of eNOS and rabies virus appears black on Negri bodies(arrow). (A): H-E stain, and (B) to (E): Immunostain counterstained with hematoxylin. Bar represents 30 μm.

(probably macrophages) in the perivascular cuffs of the rabies cases. iNOS was present in a few cells in the control brains. These results and the immunohistochemistry for NOSs in other cell types in the cerebellum are summarized in Table 1. Pre-incubation with antibody to rabies virus did not prevent a positive result with eNOS staining and vice versa. Double labeling revealed eNOS and rabies virus antigen in the same inclusion bodies (Fig. 1E), although they were not superimposed.

This is the first confirmation that eNOS is specifically localized in Negri bodies in neurons of cattle with rabies, while iNOS is expressed mainly in inflammatory cells within perivascular cuffs, as documented previously [14]. This study demonstrated differences in the distribution of the antigens of the two isoforms of NOS in the brains of cattle that were naturally infected with rabies virus.

There is a general agreement that increased expression of iNOS, by generating NO, has a detrimental effect in virus infection in the CNS. Considering the characteristics of Negri bodies, which were immunostained for viral structural protein in this study, it is possible that eNOS aggregates in the cytoskeleton in infected neurons, ultimately forming

Table 1. Immunohistochemical localization of iNOS and eNOS in the cerebellum of cattle that were naturally infected with rabies virus

	iNOS	eNOS
Molecular cell layer	–	+
Purkinje cells	+	++[a)]
Granular cell layer	–	+/–
Glial cells	–	+/–
Vascular endothelial cells	–	+[b)]
Inflammatory cells	+[c)]	–

Immunoreactivity: – (negative), +/– (occasional cells were positive), + (one third of the cells were positive), ++ (two thirds of the cells were positive).
a) Specific labeling in inclusion bodies.
b) Staining in the endothelium and adventitia.
c) Macrophages.

Negri bodies, either causally or consequently. This is further supported by the fact that eNOS is a novel Golgi-associated protein, and compartmentalization in the Golgi apparatus is necessary for the enzyme to respond to intracellular signals and produce NO [13].

Although there is a consensus that by generating NO eNOS plays a role in controlling blood flow [10], we postulated that increased eNOS expression may in part be involved in the cell death process via the generation of NO, in which neuronal death is one of the common finding in rabies virus infection [7, 8]. Further study of the precise role of eNOS in rabies virus pathogenesis is needed.

In conclusion, expression of both endothelial and inducible NOS was increased in the brains of cattle that were infected with rabies virus. Interestingly, eNOS appears to play a role in the neuropathogenesis of rabies infection, as we demonstrated the co-localization of eNOS and rabies virus nucleoprotein in the same inclusion bodies in neurons in this study. The exact role of eNOS in the neuropathogenesis of rabies virus infection remains to be determined.

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